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Altered Composition and Increased Endothelial Cell Proliferative Activity of Proteoglycans Isolated From Breast Carcinoma

PARAKAT VIJAYAGOPAL, PhD,[1]* JULIO E. FIGUEROA, MD,[2] AND EDWARD A. LEVINE, MD[3]
[1]Section of Cardiology, Department of Medicine, Louisiana State University Medical Center, New Orleans, Louisiana
[2]Section of Infectious Diseases, Department of Medicine, Louisiana State University Medical Center, New Orleans, Louisiana
[3]Section of Surgical Oncology, Department of Surgery, Louisiana State University Medical Center, New Orleans, Louisiana

Background and Objectives: Proteoglycans of the extracellular matrix are vital to the growth and evolution of malignant neoplasms. The present study determined the composition of proteoglycans isolated from paired specimens of normal breast and adenocarcinoma of the breast harvested from each patient (n = 8). The proteoglycans were then tested for their ability to stimulate endothelial cell proliferation.
Methods: Proteoglycans were isolated by extraction with 4 M guanidine hydrochloride and purified by CsCl density-gradient centrifugation. The proteoglycans were characterized and tested for their ability to simulate endothelial cell proliferation.
Results: In each case, the total proteoglycan content of the tumor was significantly greater than that of the corresponding normal tissue. The proteoglycans isolated from the carcinoma contained 32.2% (13.7/42.5) more chondroitin sulfate, 18.5% (5.6/30.2) less dermatan sulfate, and 29.6% (8.1/27.3) less heparan sulfate than did the proteoglycans of normal breast tissue. Proteoglycans from normal tissue did not stimulate endothelial cell proliferation, whereas those from malignant tissue stimulated proliferation by 1.3- to 1.5-fold.
Conclusions: These results indicate that malignant breast tissue exhibits both qualitative and quantitative changes in proteoglycan composition, which, in turn, may stimulate endothelial cell proliferation.
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KEY WORDS: breast cancer; proteoglycans; endothelial cell proliferation

INTRODUCTION

Proteoglycans (PG) are major constituents of the extracellular matrix that surrounds cells. They are complex molecules that contain one or more chains of glycosaminoglycans and oligosaccharides, covalently linked to a central protein core through serine residues. In addition to their important role in the organization and function of the extracellular matrix, PG participate in several critical biologic processes, including cell proliferation, cell-to-cell contact, and cell migration [1–3]. Because the extracellular matrix is vital to the growth and evolution of malignant neoplasms, it is highly likely that alterations in matrix molecules may profoundly influence tumor development.

Several studies have demonstrated that human tumors have altered glycosaminoglycan/PG composition. In par-

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*Correspondence to: Parakat Vijayagopal, PhD, Department of Medicine, Louisiana State University Medical Center, 1542 Tulane Avenue, New Orleans, LA 70112. Fax No.: (504) 568-2127. E-mail: pvijay@lsumc.edu
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ticular, investigators have reported increased hyaluronic acid levels in mesothelioma and Wilms tumor, reduced levels of keratan sulfate PG in human chondrosarcoma, and increased chondroitin sulfate PG levels in a variety of epithelial and mesenchymal neoplasma [4]. Little is known about the changes in extracellular matrix PG composition in breast carcinomas. Alini and Losa [5] reported that PG content of neoplastic breast tissue was twofold greater than that of nonneoplastic breast tissue due to an increase in chondroitin sulfate PG. We hypothesized that, compared with normal breast tissue, malignant breast tissue had altered PG composition and structure.

Pathological studies of breast tissues have suggested that increased neovascularization in a tumor is associated with higher rates of lymph node metastasis. Therefore, tumor angiogenesis appears to correlate with a poorer prognosis. Heparin and heparan sulfate play key roles in angiogenesis, primarily by modulating the angiogenic activity of fibroblast growth factor [6]. Also, West and Kumar [7] reported that certain degradation products of hyaluronic acid directly stimulate angiogenesis through stimulation of endothelial cell proliferation. The roles that other extracellular matrix PG play in angiogenesis are unknown. Moreover, there have been no studies evaluating the angiogenic potential of PG isolated from breast carcinoma. In the present study, we compared the PG composition of paired specimens of normal and carcinomatous breast tissue from women undergoing mastectomy and studied the mitogenic potential of the PG in endothelial cells.

MATERIALS AND METHODS

Tissues

We obtained paired normal and neoplastic breast tissues (n = 8) from the same breast of patients undergoing definitive surgical therapy for biopsy-proven adenocarcinoma of the breast. The malignant and normal nature of the tissue was confirmed by histopathological analysis for all the harvested tissues. From each patient, an aliquot of tumor and normal breast tissue was snap-frozen at −80°C until used for PG analysis. The use of the tissue was approved by the Institutional Review Board.

PG Extraction

The tissue was defatted by cold acetone extraction and PG were extracted with 4 M guanidine HCl, 50 mM sodium acetate (pH 5.8) containing protease inhibitors (100 mM ε-aminocaproic acid, 5 mM benzamidine HCl, 10 mM ethylenediamine tetraacetic acid [EDTA], 5 mM N-ethylmaleimide, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) for 24 h at 4°C with gentle stirring. After removal of the supernatant, fresh extraction buffer was added, and the tissue was extracted for an additional 24 h. Solid CsCl was added to the combined extract to a loading density of 1.4 g/ml, and the resultant mixture was centrifuged at 40,000 rpm in a Beckman 40.3 rotor for 48 h at 8°C. After centrifugation, the bottom two-thirds of the sample from each tube was dialyzed first against 50 mM sodium acetate and then against distilled water and subsequently lyophilized.

TABLE I. Patient Demographics and Staging and Hormone Receptor Data of Breast Carcinoma*

Patient	Age	Race	TNM	Stage	ER	PR	DNA
1	48	W	G2T2N0M0	II	17	2	N/A
2	48	H	G3T4N1M1	IV	0	0	Aneuploid
3	64	B	G2T2N0M0	II	0	0	Aneuploid
4	57	B	G3T2N0M0	II	23	11	Aneuploid
5	52	B	G3T2N0M0	II	2	3	Aneuploid
6	71	B	G3T2N0M0	II	>500	>500	Diploid
7	88	W	G3T4N1M0	III	2	12	N/A
8	64	B	G3T4N1M0	III	0	0	Aneuploid

*Hormone receptor measurement is in femtomole/mg cytosol protein. W, Caucasian; H, Hispanic, B, African American; TNM, tumor node metastasis (staging per the AJCC 1992 manual of staging); ER, estrogen receptor; PR, progesterone receptor; N/A, not available.

The tissue remaining after guanidine HCl extraction was digested with papain (0.5 mg/ml) in 0.1 M sodium acetate, 10 mM cystine, and 10 mM EDTA (pH 5.8) for 24 h at 65°C. Proteins were removed from the papain digest by precipitation with trichloroacetic acid (TCA) (10%), and total glycosaminoglycans were isolated by precipitation with cetylpyridinium chloride [8].

Analysis of PG/glycosaminoglycans

The PG/glycosaminoglycan content of guanidine HCl-extracted and papain-digested materials was determined by uronic acid analysis [9]. The composition of individual glycosaminoglycans was determined by digestion with chondroitinase ABC, chondroitinase AC [10], and nitrous acid treatment [11].

Endothelial Cell Proliferation Assay

We used [^{3}H]thymidine incorporation assay to determine the effect of PG from normal and malignant breast tissues and individual glycosaminoglycans (chondroitin 4-sulfate from shark cartilage, dermatan sulfate from pig skin, and heparin from beef lung; all three were obtained from Sigma Chemical Co., St. Louis, MO). Briefly, human umbilical vein endothelial cells were plated (5×10^4 cells/well in 24-well culture plates) in RPMI 1640 containing 10% fetal bovine serum (FBS). After 48 h, the cells were switched to serum-free RPMI. PG (10 μg uronic acid/ml) were added to the cultures after 24 h, and the incubation was continued. Serum (1% FBS) was added to the cultures after 24 h, and the cells were incubated at 37°C for 24 h. At 6 h before the termination of the experiment, [^{3}H]thymidine (1 μCi/well) was added to the cultures, and ^{3}H radioactivity in the cells was assayed.

TABLE II. Proteoglycan Content of Normal and Malignant Breast Tissues†

Patient	Normal tissue: GudHCl extract/total extract (μg uronic acid/g dry tissue)	Malignant tissue: GudHCl extract/total extract (μg uronic acid/g dry tissue)
1	11.9/13.2 (90)	131.7/144.3 (91.3)
2	14.9/17.3 (86)	52.8/59.3 (89)
3	16.9/19.2 (88)	164.6/187.0 (88)
4	12.3/14.4 (85)	137.3/148.9 (92.2)
5	13.2/15.1 (87)	161.7/190.2 (85)
6	15.3/16.6 (92)	274.9/302.1 (91)
7	16.3/18.3 (89)	76.8/90.4 (85)
8	12.6/14.5 (87)	171.5/188.4 (91)
Mean ±SD	14.2 ± 1.9/16.1 ± 2.1	146.4 ± 67.2*/163.8 ± 73.6*

†Values in parentheses are percentage extracted with guanidine HCl.
GudHCl, guanidine HCl; total extract = GudHCl extract + papain digest.
*Significantly higher than the values for the corresponding normal tissue; $P < 0.0001$.

Statistical Analysis

Statistical analysis was performed by unpaired, two-tailed *t*-test.

RESULTS

Patient Characteristics

Table I shows patient demographics and staging and hormone receptor data of breast carcinoma. The age of the patients was within a range of 48–88 years. All carcinomas were of the infiltrating ductal type, and either intermediate (G2) or high (G3) histologic grade. DNA content was stratified as either diploid or aneuploid. Patients 1, 3, and 4 had lumpectomy, patient 2 underwent radical mastectomy, and all other patients had modified radical mastectomy. Patients 2, 7, and 8 are known to have died of disease, with the other patients free of disease with a mean follow-up of 35 months.

PG Composition of Normal and Neoplastic Breast Tissues

Table II shows the PG content of normal and malignant breast tissues. Guanidine HCl extraction removed almost equal amounts of PG from both tissues (85–92%). However, in each case, the total PG content (guanidine HCl extract + papain digest) of tumor tissue was 3- to 18-fold greater than that of the corresponding normal tissue. On average, the guanidine HCl extract of tumor tissue contained 10-fold more PG than did normal breast tissue (146.4 ± 67.8 vs. 14.2 ± 1.9, μg uronic acid/g tissue; $P < 0.0001$).

After CsCl centrifugation, the bottom two-thirds of the gradient contained 90–93% of the PG extracted with guanidine HCl from all tissues. Table III shows the glycosaminoglycan composition of the PG in the ultracentrifuge fraction. Compared with normal tissues, the tumor tissues contained 32.2% (13.7/42.5) more chondroitin sulfates, 18.5% (5.6/30.2) less dermatan sulfate, and 29.6% (8.1/27.3) less heparan sulfate.

TABLE III. Glycosaminoglycan Composition of Proteoglycans From Normal and Malignant Breast Tissues†

Tissue	Glycosaminoglycans: Chondroitin sulfate (%)	Dermatan sulfate (%)	Heparan sulfate (%)
Normal (n = 8)	42.5 ± 3.2	30.2 ± 2.8	27.3 ± 4.5
Tumor (n = 8)	56.2 ± 2.5*	24.6 ± 3.6*	19.2 ± 3.8*

†Values are the mean ± SD.
*Significantly different from the corresponding normal tissue; $P < 0.001$.

Effect of Proteoglycans on Endothelial Cell Proliferation

We used the guanidine HCl extracted total PG from four patients in cell proliferation assay. The PG samples from the other patients were not available. The results show that PG from normal breast tissues did not stimulate endothelial cell proliferation (Table IV). However, at an equivalent concentration (10 μg uronic acid/ml), PG from the corresponding malignant tissues stimulated cell proliferation by 1.3- to 1.5-fold. In a group comparison, the PG from carcinoma showed significant stimulation of endothelial cell proliferation compared with the PG from normal tissue (56,625 ± 3,880 vs. 36,175 ± 1,681, ^{3}H-dpm ± SD, $P < 0.0001$).

In order to determine whether glycosaminoglycans isolated from nonhuman sources affected endothelial cell growth, we tested chondroitin 4-sulfate from shark cartilage, dermatan sulfate from pig skin, and heparin from beef lung in the endothelial cell proliferation assay. As shown in Figure 1A (filled circle), heparin seemed to decrease endothelial cell proliferation in a dose-dependent manner. At a concentration of 10 μg/ml, der-

TABLE IV. Effect of Proteoglycans From Normal and Malignant Breast Tissues on Endothelial Cell Proliferation†

Patient variable	Tissue type	EC proliferation ([^{3}H]thymidine incorporated, dpm)
Control	None	37,880
1	Normal	38,500
	Tumor	60,870
3	Normal	36,090
	Tumor	55,530
4	Normal	35,580
	Tumor	58,290
6	Normal	34,530
	Tumor	51,810
Mean:	Normal	36,175 ± 1,681
	Tumor	56,625 ± 3,880*

†Endothelial cells (EC) were plated (5×10^4 cells/well) in RPMI 1640 containing 10% fetal bovine serum. After 48 h, the cells were switched to serum-free RPMI. Proteoglycans (10 μg uronic acid/ml) were added to the cultures after 24 h, and the incubation was continued. Serum (1% fetal bovine serum) was added to the cultures after 24 h, and the cells were incubated at 37°C for 24 h. At 6 h before termination of the experiment, [^{3}H]thymidine (1 μCi/well) was added to the cultures, and ^{3}H radioactivity in the cells was assayed.
*Significantly greater than normal; $P < 0.0001$.

matan sulfate stimulated endothelial cell proliferation (Fig. 1B). At this concentration chondroitin 4-sulfate was also slightly stimulatory (Fig. 1A, filled triangle). At higher concentrations both glycosaminoglycans lost their proliferative action.

DISCUSSION

Our study shows that the PG content and composition in human breast adenocarcinoma tissue are altered compared with their content and composition in normal breast tissue. These results confirm the earlier observations of Alini and Losa [5], although the source of tissues analyzed in our study and theirs is not identical. We used malignant and normal breast tissues harvested from the same patient, while Alini and Losa obtained these tissues from different subjects. However, it is obvious from the results that, regardless of the source of the tissues, PG metabolism is significantly altered in breast cancer.

The tumor tissue contained significantly more PG than did the normal tissue ($P < 0.0001$). This is consistent with the increased PG content reported for other human tumors [4] and may result from the interactions between the malignant cells and the host cells [4]. Most likely, the increased PG content of breast tumors stimulates tumor progression by providing a PG-rich matrix in which the tumor cells grow rapidly and proliferate.

A comparison of the glycosaminoglycan composition of PG isolated from normal and neoplastic breast tissues indicates that tumor PG contain more chondroitin sulfates and less dermatan sulfate and heparan sulfate. Earlier studies have reported elevated levels of chondroitin sulfate PG in tumors of the lung [12,13], liver [14,15],

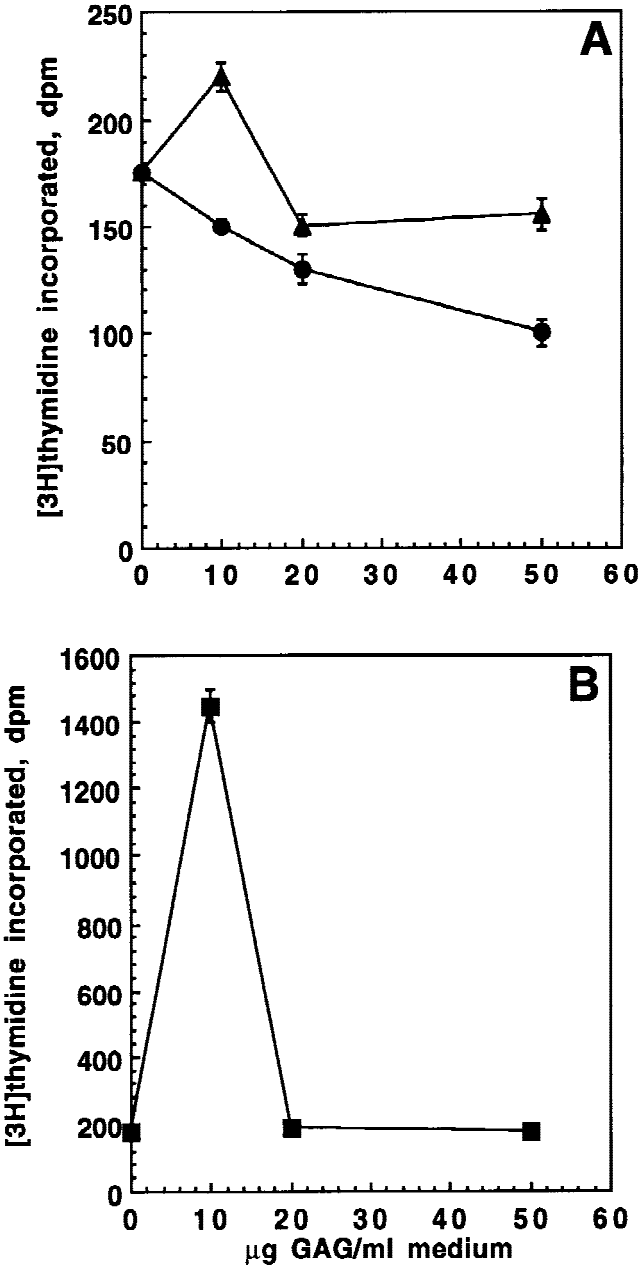

Fig. 1. Effect of glycosaminoglycans on endothelial cell proliferation. Endothelial cells were plated (5×10^4 cells/well) in RPMI 1640 containing 10% fetal bovine serum. After 48 h, the cells were switched to serum-free RPMI. The indicated concentrations of glycosaminoglycans were added to the cultures after 24 h, and the incubation was continued. Serum (1% fetal bovine serum) was added to the cultures after 24 h, and the cells were incubated at 37°C for 24 h. At 6 h before the termination of the experiment, [^{3}H]thymidine (1 μCi/well) was added to the cultures, and ^{3}H radioactivity in the cells was assayed. **A:** Heparin, ●; chondroitin 4-sulfate, ▲. **B:** Dermatan sulfate. dpm, disintegrations per minute; GAG, glycosaminoglycans. Each data point represents the mean ± SEM of three separate experiments, each performed in triplicate. Absent error bars indicate SEM values smaller than the drawn symbols.

and colon [16–18]. An increase in chondroitin sulfate PG in malignant breast tissue may promote further tumor progression since previous studies have found that this PG promotes the growth of tumor cells in vitro [19] and in vivo [20]. The loss of heparan sulfate PG can contribute to tumor progression in two ways. First, depletion of this PG from the basement membrane will facilitate tumor invasion. In addition, because heparan sulfate is a known inhibitor of smooth muscle cell proliferation [21], a decrease in this PG removes a natural inhibitor of proliferation and thus will favor tumor cell growth. The significance of dermatan sulfate depletion in malignant breast tumor is unclear. It may be a simple response to the increase in chondroitin sulfate. Alternatively, this may have broader implications. Decorin, a dermatan sulfate PG, is involved in collagen fibrillogenesis, and a decrease in decorin may influence this process. Impaired collagen fibril formation, in turn, may aid tumor invasion.

An interesting finding of our study is that breast tumor PG are potent stimulators of endothelial cell proliferation. In contrast, PG isolated from normal breast tissue exhibit virtually no proliferative properties. At present, we cannot attribute the proliferative action to any specific PG species from the tumor because we did not study individual PG for their proliferative action. Heparin and heparan sulfate play key roles in angiogenesis, primarily by modulating the angiogenic activity of basic fibroblast growth factor [6]. However, PG from breast tumor contain less heparan sulfate than do the PG from normal breast tissue. Therefore, the proliferative action of the tumor PG cannot be attributed to heparin or heparan sulfate and must be attributable to chondroitin sulfate and/or dermatan sulfate content. In our study, dermatan sulfate from pig skin stimulated endothelial cell proliferation and, therefore, PG containing this glycosaminoglycan may also promote proliferation. However, the tumor PG contained relatively less dermatan sulfate than did the PG from normal tissue. This finding suggests that the proliferative action of breast cancer PG may be due to their increased chondroitin sulfate content. These PG may stimulate endothelial cell proliferation by several mechanisms: a direct receptor-mediated induction, modulation of other angiogenic factors already present in the system, and direct activation of endothelial cells resulting in the secretion of proteases that degrade basement membrane. Regardless of the mechanism by which breast cancer PG stimulate endothelial cell proliferation, they can contribute to angiogenesis, which is central to cancer cell growth and metastasis.

CONCLUSIONS

Our study demonstrates that malignant breast tissue exhibits both qualitative and quantitative changes in PG composition—changes that result in marked stimulation of endothelial cell proliferation. Further research to understand the mechanism of altered PG metabolism as well as PG-stimulated endothelial cell proliferation could lead to novel approaches for breast cancer treatment.

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